UNIVERSITI PUTRA MALAYSIA

IN VITRO EXPRESSION OF THE CTXB TOXIN GENE TOWARDS THE DEVELOPMENT OF A DNA VACCINE AGAINST CHOLERA

SYAHRILNIZAM ABDULLAH

FPSK (M) 2001 12

IN VITRO EXPRESSION OF THE CTXB TOXIN GENE TOWARDS THE DEVELOPMENT OF A DNA VACCINE AGAINST CHOLERA

By

SYAHRILNIZAM ABDULLAH

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Medicine and Health Sciences Universiti Putra Malaysia

January 2001

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science.

IN VITRO EXPRESSION OF THE CTXB TOXIN GENE TOWARDS THE DEVELOPMENT OF A DNA VACCINE AGAINST CHOLERA

By

SYAHRILNIZAM ABDULLAH

January 2001

Chairman: **Dr. Rozita Rosli, Ph.D**

Faculty: **Medicine and Health Sciences**

The complete eradication of cholera is an unachievable goal because it is now firmly established that there are environmental reservoirs for *Vibrio cholerae*. Although there are effective treatments for this disease, they are expensive and impractical in time of epidemic. All these points lead to the fact that the development of a safe, cheap and efficient vaccine is probably the best solution to the problem. A variety of strategies have been employed to create better vaccines against cholera but these traditional vaccines produced still suffer from a number of inherent drawbacks. Therefore, a new type of cholera vaccine is being proposed which may retain all the positive aspects of the existing vaccines while avoiding their shortcomings. It belongs to a new generation of vaccines termed DNA vaccines. The development of this vaccine has been revolutionized by the finding that antigen-encoding DNA plasmids can be used to induce cellular and humoral immune responses against immunogenic determinant. In this study, the focus is on the *ctxB* gene, the gene encoding the B subunit cholera toxin as a potential candidate for a vaccine against cholera. The *ctxB* gene is required for the binding of the Cholera Toxin (CT) to the eukaryotic cell and facilitates the entry of the active toxin (CTXA) into the host cell which in turn, produces the profuse diarrheal symptom. The *ctxB* gene has been successfully cloned in an expression vector, pVax,

and proven to be in the correct orientation by PCR and sequencing. Subsequently, the B subunit toxin was expressed *in vitro* in the pVax/*ctxB* using COS-7 cells by a non-liposomal lipid Effectene™ (Qiagen) method, 90 hours post transfection. The results from the studies indicate that the DNA plasmid carrying the *ctxB* gene (pVax/*ctxB*) was able to use the cell's transcription and translation machineries to produce the required antigen.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

EKSPRESI TOKSIN *CTXB IN VITRO* UNTUK PEMBANGUNAN VAKSIN DNA UNTUK MENGATASI TAUN (KOLERA)

Oleh

SYAHRILNIZAM ABDULLAH

Januari 2001

Pengerusi: **Dr. Rozita Rosli, Ph.D**

Fakulti: **Perubatan dan Sains Kesihatan**

Eradikasi taun adalah masalah yang tidak dapat diselesaikan kerana adanya penyimpanan alam sekeliling bakteria *Vibrio cholerae*. Walaupun perubatan yang efektif telah dihasilkan untuk penyakit ini, ianya adalah mahal dan tidak praktikal pada masa epidemik. Oleh kerana itu, vaksin yang selamat, murah dan mujarab adalah perlu untuk mengatasi masalah tersebut. Pelbagai cara telah digunakan untuk mencipta vaksin yang lebih baik tetapi vaksin-vaksin tersebut masih lagi mempunyai kekurangan. Oleh itu, vaksin taun jenis baru telah dicadangkan dimana segala aspek-aspek positif vaksin yang telah ada dikekalkan manakala kekurangannya diketepikan. Vaksin generasi baru ini dipanggil vaksin DNA. Penciptaan vaksin ini telah dipercepatkan dengan penemuan dimana plasmid DNA yang mengandungi antigen boleh mengakibatkan tindak balas imuniti "cellular" dan "humoral". Dalam kajian ini, gen *ctxB*, iaitu gen untuk toksin B kolera, menjadi fokus sebagai calon yang sesuai untuk penciptaan vaksin DNA untuk mengatasi taun. Gen *ctxB* diperlukan untuk pertambatan

toksin kolera (CT) kepada sel "eukaryote" dan menyebabkan kemasukan toksin A (CTXA) yang lebih aktif ke dalam sel lalu menghasilkan simptom cirit-birit taun. Gen *ctxB* ini telah bejaya diklon ke dalam pVax dan dibuktikan berada pada kedudukan yang betul melalui analisis Rantaian Reaksi Polimerase dan penjujukan DNA. Kemudian, toksin B telah diekspresikan daripada pVax/*ctxB* dengan menggunakan sel COS-7 dan kaedah lipid bukan liposom Effectene™ (Qiagen), 90 jam selepas transfeksi. Keputusan ujikaji ini menunjukkan bahawa plasmid yang mengandungi gen *ctxB* tersebut (pVax/*ctxB*) boleh melalui proses transkripsi dan translasi untuk menghasilkan antigen yang dikehendaki.

ACKNOWLEDGEMENTS

In the name of Allah, the Beneficent, the Merciful

Praise be to Allah, Lord of the worlds, for thee (alone) we worship and thee (alone) we ask for help. And praise be upon Muhammad s.a.w whose guidance has led us to the path that God has favoured.

The idea for this project originated from Dr. Rozita Rosli and Dr. Mariana Nor Shamsudin from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. I am indebted to them for accepting me as a post-graduate student and entrusted me in making their idea a reality.

Special thanks to Dr. Rozita Rosli for her invaluable advice, insights, ideas, patience, attention and constant help which have been the main factor over the success of this project. And to my co-supervisors, Dr. Mariana Nor Shamsudin and Associate Professor Dr. Harcharan Singh Sidhu, for their wise guidance and suggestions during this study, I am deeply grateful.

I am lucky to have wonderful colleagues at the Molecular Genetics Laboratory; Roslaini Majid, Thilakavathy, Sim Sze Kiat, Lama Hamadneh, Sharizah Alimat, Shaban Awidat and particularly Nurmawati Syakroni (who has helped me in cell culturing) and Norshariza Nordin (who has aided me in Molecular Biology techniques). Special thanks to all the medical and biomedical students that have worked in this lab. I thank the colleagues and staffs of Dr. Mariana's Microbiology Laboratory for their special help.

The period of this project has overlapped with a time of considerable pressure and unusual stress from other sources. I was most fortunate in this same period to have personal support and friendship from the people I have already mentioned, as well as from the staff of Haematology Department (Institute for Medical Research, Malaysia), Syahin, Mieza, and my brothers at Seni Silat Setia Bakti. To my special friend, Sharifah Suraya, thank you for adding more spices to my life.

I am grateful to my family for their help, understanding, emotional and financial support during the whole process of the project and writing. Finally, I am most grateful to Norana Johar who has inspired this work, and whose affection and laughter still appear in my dreams. Thank you for showing me the meaning of life.

May GOD Bless You All.

I certify that an Examination Committee met on 15th January 2001 to conduct the final examination of Syahrilnizam Abdullah on his Master of Science thesis entitled " *In Vitro* Expression of the CTXB Toxin Gene towards the Development of a DNA Vaccine against Cholera" in accordance with Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

WAN OMAR ABDULLAH, Ph.D.
Professor
Department of Clinical Sciences Laboratory
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

ROZITA ROSLI, Ph.D.
Department of Human Growth and Development
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

MARIANA NOR SHAMSUDIN, Ph.D.
Department of Clinical Sciences Laboratory
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

HARCHARAN SINGH SIDHU, Ph.D.
Department of Clinical Sciences Laboratory
Faculty of Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

MOHD GHAZALI MOHAYIDIN, Ph.D.
Professor
Deputy Dean of Graduate School
Universiti Putra Malaysia

Date: 22 FEB 2001

This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

MOHD. GHAZALI MOHAYIDIN, Ph.D.
Professor
Deputy Dean of Graduate School
Universiti Putra Malaysia

Date:

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

SYAHRILNIZAM ABDULLAH

Date: January 15, 2001

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE/*ace*	Accessory Cholera Enterotoxin
APC	Antigen Presenting Cells
ATCC	American Type Culture Collection
CBER	Centre of Biologics Evaluation and Research
CEP/*cep*	Core Encoded Pilin
CGAT	Centre for Gene Analysis and Technology
CIAP	Calf Intestinal Alkaline Phosphate
CMV	Cytomegalovirus
CO_2	Carbon Dioxide
CTL	Cytotoxic T Lymphocytes
CTX/*ctx*	*Vibrio*
Da	Daltons
DC	Dendritic Cells
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FDA	Food and Drug Administration
FITC	Fluorescein Isothiocynate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor

HA	Hemagglutinin
HbsAg	Hepatitis B Surface Antigen
HCl	Hydrocloric Acid
HIV	Human Immunodeficiency Virus
ID	Intradermal
IFA	Immunofluorescein Assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Intramuscular
IP	Intraperitonial
IPTG	Isopropyl-β-D-thiogalactoside
IV	Intravenous
KAc	Potassium Acetate
KCl	Potassium Chloride
LB	Luria Bertani
LPS	Lipopolysaccharides
LT	Heat Labile Enterotoxin
MCS	Multiple Cloning Sites
$MgCl_2$	Magnesium chloride
MHC	Major Histocompatibility Complex
MSHA	Maltose-Sensitive Hemagglutinin
MW	Molecular Weight
NaCl	Sodium Chloride

NaOH	Sodium Hydroxide
OD	Optical Density
ORF	Open Reading Frame
ORS	Oral Rehydration Salts
PBS	Phosphate buffered Saline
PRR	Pattern Recognition Receptor
PVDF	Polyvinylidane Difluoride
RNA	Ribonucleic Acid
RSV	Raos Sarcoma Virus
SDS	Sodium Dodecyl Sulfate
SSC	Standard Saline Citrate
TCBS	Thiosulphate-Citrate-Bile salts Sucrose agar
TCP	Toxin Coregulated Pilus
TEM	Transmission Electron Microscopy
Th	Helper T Cells
UKM	Universiti Kebangsaan Malaysia
UM	Universiti Malaya
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VPI	*Vibrio cholerae* Pathogenicity Island
WHO	World Health Organization
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-Galactoside
ZOT/*zot*	Zonula Occludens Toxin

CHAPTER 1

INTRODUCTION

In 1854, when a cholera epidemic erupted in India and traveled west to the town of London, a renowned anesthesiologist, Dr. John Snow dedicated himself to discover the source of the devastating disease. His work took him from the upper-class residence to the poor, marking the house of the people stroked by the disease on a map. Noticing that the cases centered on a public water pump at Broad Street, South London, Snow persuaded the authorities to remove the pump handle which forced the residents to go elsewhere for a source of drinking water. As soon as the water from the well was no longer being used, the cases of cholera declined dramatically.

Dr. John Snow's work earned him a legendary status in the field of public health and epidemiology. His essay titled "On the Mode of Communication of Cholera" published in 1855 (1), was the first treatise in which he set forth his observations and recommendations on instances of cholera and measures to put this disease arrest.

Yet, 145 years later, we are still plagued by this dreadful disease. A half million cases have been reported to the World Health Organization (WHO) in the last two years with 20,000 deaths. The reported overall case-fatality rate (CFR) for 1999 has remained stable at 3.6%, in which the Asian continent showed a 61% increase compared to 1998. To make matters worse, the number of deaths notified to WHO due to this disease has also doubled. As for Malaysia, a 535 case-fatality rate was reported to WHO in 1999

with no death (2). Although there is no mortality, this number signifies that cholera is one of the major gastrointestinal diseases in this country.

The significance of this disease has prompted scientists to sequence the whole genome of *Vibrio cholerae*, the aetiological agent for cholera. The complete sequence of both the chromosomes of the cholera pathogen has been published recently (3) and it is hoped that this knowledge will help us understand the complete molecular description of this pathogen and formulate newer strategies to eradicate this disease.

Despite the suggestion by Dr. John Snow, it is clear that trying to provide uncontaminated water supply and educating the public on the preventive measures only are not enough to control the transmission of cholera, especially in the developing world. Even though we now have better treatments for cholera, such as antibiotics and oral rehydration salts, compared to during the time of Dr. Snow's, these remedies are not practical. Antibiotics, for instance, are quite costly and their excessive use can promote the development of a new antibiotic resistance bacterial strain, which will defeat the purpose of the treatment.

The best alternative is to provide a cholera vaccine that will immunize the public before an outbreak occurs. Several cholera vaccines have been created, yet they are not effective and potent enough. It has been noted by WHO that these traditional vaccines evoke protection against illness in only about 50% of individuals immunized and they last only 3-6 months, and are even less effective in younger children (4). Clearly, these traditional vaccines have not delivered the response we require.

Fortunately, due to the unmet needs of old and new epidemics of infectious diseases, as well as the advent of molecular biology, a new era of vaccinology has been stimulated. This novel approach employs plasmid DNA, engineered to express one or more genes of the pathogen in mammalian cells. The proteins expressed lead to a stronger and persistent cell-mediated and humoral immune response compared to the conventional and the recombinant vaccines. This unique approach to immunization, termed DNA vaccination, may overcome deficits of the traditional antigen-based methods and provide safe and effective prophylactic and therapeutic vaccines. Since it is easy to produce in mass once it has been manufactured, this DNA vaccine will be much cheaper than the conventional vaccine.

In this study, we manipulated and used the knowledge in genetic engineering, recombinant DNA technology and immunology to design a DNA vaccine against the disease cholera. The vaccine candidate consists of the *ctxB* gene, the gene encoding the B subunit enterotoxin of the *Vibrio cholera.* It is hoped that the B subunit of this cholera toxin when expressed will establish adequate levels of antibody and a primed population of cells, which would rapidly expand in numbers upon second contact with the "real" pathogen.

Objectives

Thus, the objectives of this study are: (1) to identify and amplify the DNA sequence of *Vibrio cholera* B subunit cholera toxin from local cholera strains, (2) to confirm the DNA sequence by probing with an identified target DNA sequence (oligonucleotide) and by sequencing the amplified DNA product, (3) to clone the sequence in an appropriate mammalian expression vaccine vector, and finally (4) to assess the expression of the product in mammalian cell culture.

CHAPTER II

LITERATURE REVIEW

Cholera

The History

Cholera has afflicted humans for more than 2,000 years. It has smoldered in an endemic fashion on the Indian subcontinent for centuries. The symptoms were mentioned as long ago as Hippocrates and even earlier in Sanskrit writings. Epidemic cholera was described in 1563 by Garcia del Huerto, a Portuguese physician at Goa, India. The mode of transmission of cholera by water was proven in 1849 by John Snow, a London physician. In 1883, Robert Koch successfully isolated the cholera *vibrio* from the intestinal discharges of cholera patients and proved conclusively that it was the agent of the disease.

The first long-distance spread of cholera to Europe and the Americas began in 1817 and by the early 20th century, six waves of cholera had spread across the world in devastating epidemic fashion. Since then, until the 1960s, the distribution of the disease contracted, remaining present only in southern Asia. In 1961, the "*El Tor*" biotype (distinguished from Classic biotypes by the production of hemolysins) re-emerged to produce a major epidemic in the Philippines and to initiate a seventh global pandemic.